



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US97/20364 <b>(22) International Filing Date:</b> 7 November 1997 (07.11.97)  <b>(30) Priority Data:</b> 60/030,718                      8 November 1996 (08.11.96)      US 60/054,533                      4 August 1997 (04.08.97)        US  <b>(71) Applicant:</b> OKLAHOMA MEDICAL RESEARCH FOUNDATION [US/US]; 825 N.E. Thirteenth Street, Oklahoma City, OK 73104 (US).  <b>(72) Inventors:</b> ESMON, Charles, T.; 5800 North Stonewall, Oklahoma City, OK 73111 (US). GU, Jian-Ming; 3630 North Villa Avenue #2, Oklahoma City, OK 73112 (US).  <b>(74) Agent:</b> PABST, Patrea, L.; Amall Golden & Gregory, LLP, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS		
<b>(57) Abstract</b> <p>The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been demonstrated to include a region which results in selective expression in endothelial cells, between -1 and -220 based on the positions relative to the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expression in large vessel endothelial cells, as opposed to all endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin or serum or to effect selective expression in endothelial cells generally or preferentially in endothelial cells of the large blood vessels. The gene control elements include elements responsive to environmental stimuli (either thrombin or serum); and information to determine distribution of the desired protein expression (large vessels). Therapeutic strategies include the use of the minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of gene therapy where systemic distribution is desired; the use of a promoter including an environmental stimuli response element(s), for use in delivery of agents whose expression should be increased during times of increased thrombin/platelet activation or during regional trauma; the use of the minimal promoter including an environmental stimuli response element and the element directing expression to large vessel endothelium, where a response to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal promoter and element directing expression to large vessel endothelium, where expression is specifically targeted to large vessel endothelium but is not increased in response to any particular stimuli.</p>		

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## ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS

### Background of the Invention

The United States government has certain rights in this invention  
5 by virtue of National Heart, Lung and Blood Institute of the Institutes of  
Health grant No. P01 HL54804 to Charles T. Esmon.

The present invention is generally in the area of targeting and  
regulation of expression of recombinant gene constructs incorporating  
regulatory elements present in the promoter of an endothelial cell protein  
10 C/activated protein C receptor.

Atherosclerosis and most other vascular disease primarily occur in  
large vessels. Endothelial cells are a primary defense mechanism against  
cellular infiltration and thrombosis. Abnormal function of the endothelial  
cells contribute to myocardial infarction (MI), stroke and the development  
15 of atherosclerotic plaque. The delivery of proteins or protein expression  
inhibitors, directly or via gene therapy, specific to large vessel endothelial  
cells, is one means for addressing these clinical conditions. For example,  
the anti-thrombotic potential of endothelium can be increased by  
delivering agents that prevent thrombosis, such as thrombomodulin,  
20 heparin proteoglycans, tissue factor pathway inhibitor (TFPI, a potent  
inhibitor of the tissue Factor-Factor VIIa-Factor Xa complex), etc.  
Fibrinolytic activity can be increased by overexpression of tissue  
plasminogen activator (tPA) or urokinase. Expression of adhesion  
molecules such as P-selectin or ICAMs can be inhibited to minimize or  
25 decrease the probability of atherosclerotic plaque rupture.

Targeting endothelial cells non-specifically is often inadequate.  
Since more than 95% of endothelial cells are in the capillaries, any  
therapy directed toward endothelial cells per se runs the risk of systemic  
complications. One must be confident that the gene expression is limited  
30 to the desired cells when using a gene therapy approach.

It is therefore an object of the present invention to provide means and methods for selective expression of genes, especially in endothelial cells, and even more specifically in large vessel endothelial cells.

It is a further object of the present invention to provide means and  
5 methods for selective expression of genes in response to specific stimuli.

### Summary of the Invention

The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been demonstrated to include a region which results in  
10 selective expression in endothelial cells, between -1 and -220 based on the positions relative to the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expression in large vessel endothelial cells, as opposed to all endothelial cells, located between -700 and -1080  
15 (nucleotides 2270 to 2840 of SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A serum  
20 response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1).

The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin or serum  
25 or to effect selective expression in endothelial cells generally or preferentially in endothelial cells of the large blood vessels. The gene control elements include elements responsive to environmental stimuli (either thrombin or serum); and information to determine distribution of the desired protein expression (large vessels). Therapeutic strategies  
30 include the use of the minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of gene therapy where

systemic distribution is desired: the use of a promoter including an environmental stimuli response element(s). for use in delivery of agents whose expression should be increased during times of increased thrombin/platelet activation or during regional trauma; the use of the minimal promoter including an environmental stimuli response element and the element directing expression to large vessel endothelium, where a response to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal promoter and element directing expression to large vessel endothelium, where expression is specifically targeted to large vessel endothelium but is not increased in response to any particular stimuli.

#### Brief Description of the Drawing

Figure 1 is a comparison of the nucleotide sequences of the murine EPCR and human EPCR promoters (SEQ. ID Nos. 1 and 2, respectively).

Figure 2 is a graph of relative levels of expression (relative luminescent units) for mP3340, mP1120, mP700, mP350 and control SV40, in bovine endothelial cells (large vessel endothelial cells), rat heart endothelial cells, mostly capillary cells (small vessel endothelial cells), and 293 kidney cells (non-endothelial cells).

Figure 3 is a schematic of the constructs transfected into bovine aortic (large vessel) endothelial cells, graphing the relative levels of expression (relative luminescent units) for mP1120, mP550, mP350 (AP1 mutant), mP350 (deletion from 280 to 160), mP280, mP220, mP180, mP160, and mP80, with the pGL3 control.

Figure 4 is a schematic of the promoter. The top line indicates the structure of the promoter from -220 to -180, which includes the transcription control elements required for constitutive expression in endothelial cells. AP-4 and SP-1 are known promoter elements that bind proteins that control gene expression. The bottom line is a schematic representation of the EPCR promoter showing the locations of the large

vessel specific element between -1080 and -700 ("C"), the element which includes the sequence responsible for thrombin induction ("B"), the endothelial specific region ("A"), and the EPCR encoding element. SP-1, AP-1 and AP-4 are known promoter elements which bind proteins involved in transcription control.

### Detailed Description of the Invention

Specific targeting of expression of desired genes can be achieved through the selection and use of the regulatory sequences described herein in detail, isolated from the protein C receptor (EPCR). The protein C receptor is the first protein identified and reported with these properties. It is expressed in high levels exclusively in large vessels, and the expression levels decrease with vessel size, until there is little to no expression detectable in capillaries.

#### The EPCR Regulatory Sequences

The endothelial cell protein C binding protein (referred to herein as "EPCR") was cloned and characterized, as described in PCT/US95/09636 "*Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor*" Oklahoma Medical Research Foundation. The protein was predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type I transmembrane protein. The protein binds with high affinity to both protein C and activated protein C ( $K_d = 30$  nM), which is a naturally occurring anticoagulant, and is calcium dependent.

Following identification and cloning of the endothelial cell protein C receptor (EPCR), it was determined that the EPCR was down regulated in cultured endothelial cells by  $\text{TNF}\alpha$ . To determine the physiological relevance of this finding, EPCR mRNA levels in rats and mice challenged with  $\text{LD}_{95}$  levels of endotoxin were examined. Surprisingly, in response to endotoxin infusion, EPCR message rose within three hours to about four fold the basal level and remained elevated for twelve hours, then

returning toward baseline at 24 hours. The rapid response suggested that a factor generated by endotoxin infusion could upregulate EPCR expression. Since thrombin is known to be one of these factors, rat microvascular cells in culture were treated with thrombin (0.1 units/ml). The cells exhibited a  
5 three to four fold increase in EPCR mRNA levels within three hours relative to control cells.

Physiologically, these results showing elevated mRNA levels three hours after exposure to thrombin, which begins to decline after twelve hours to baseline levels by 24 hours, are important since they suggest that  
10 thrombin plays a direct *in vivo* role in upregulation of EPCR expression. High level EPCR expression could contribute to the decrease observed in protein C levels during acute inflammatory response syndromes.

The gene encoding EPCR including the promoter region was then isolated from a murine genomic library, using the DNA encoding murine  
15 EPCR as a probe. A human genomic library was similarly screened with the DNA encoding human EPCR to isolate the promoter for the human EPCR. Analysis of the promoter revealed a thrombin response element. Gel shift assays revealed that thrombin treatment induced at least one factor that binds specifically to this promoter region. Further analysis yielded the sequence of  
20 the thrombin responsive element. This element can be used to increase selective expression in response to thrombin. The promoter is also selective in expression, with the EPCR being selectively expressed more in large vessel endothelial cells when most of the entire promoter is present, including the beginning region. When a shorter portion of the promoter is  
25 present, there is expression in all endothelial cells. These results are consistent with a repressor being present in the first part of the promoter which suppresses expression in capillary endothelial cells.

Referring to Figures 1A and 1B and SEQ. ID Nos 1 (the murine EPCR promoter) and 2 (the human EPCR promoter), the 5' regulatory  
30 sequences of the EPCR includes a transcription initiation promoter specific to endothelium contained in -1 to -220 (nucleotides 3130 to 3350 of SEQ. ID

No. 1) (referred to for ease of reference as "A"), a control element responsive to thrombin (CCCACCCC) (SEQ. ID No. 3) located between -337 and -345 in the murine promoter (nucleotides 3007 to 3014 of SEQ ID No. 1) and between -360 and -368 in the human promoter (nucleotides 2722 to 2729 of SEQ. ID No. 2) (referred to as "B"), a serum response element located between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1) (referred to as "D"), and a large vessel expression element located between -1080 and -700 (nucleotides 2270 to 2840 of SEQ. ID No. 1) (referred to as "C"). The latter directs expression primarily to large vessels such as aorta, coronary arteries, arteries and veins, rather than to capillaries.

Figures 1A and 1B are a comparison of the sequences from the murine and human promoters, demonstrating that they are highly homologous. It is understood that the equivalent regions from the promoters of EPCR from other species could be used to achieve the same type of expression, and that sequences from different species could be used in combination, for example, A from the murine promoter and C from the human promoter.

#### Expression Constructs

These regulatory elements can be used alone or in various combinations, as demonstrated by the examples, to determine where and to what extent expression is obtained, both *in vitro* and *in vivo*. Region A can drive endothelial cell specific expression. Adding to this region A, region C would result in expression occurring primarily in large vessels. Adding region B to these regions A and C, results in a thrombin response - i.e., expression is increased by exposure to thrombin, as would occur in a patient during initiation of coagulation or an inflammatory response.

The regulatory sequences can be inserted into vectors for expression using standard recombinant techniques.

#### The Regulatory Elements are useful as Reagents

The nucleotide sequences are important as hybridization probes, in selected expression of recombinant proteins other than EPCR, in increasing expression of recombinant proteins by exposure of the



encoding construct to thrombin, and in design and screening of drugs and diagnostics for therapeutic and research purposes.

#### Methods of Treatment

The constructs are particularly useful in gene therapy. The elements can be used to regulate expression of a gene encoding an important protein, or a biologically active nucleic acid molecule such as antisense, triplex forming molecules, ribozymes, and guide sequences for RNAase P which can be used to mutate or stop transcription of a particular gene. Examples of gene targeting include expression of thrombomodulin (TM), EPCR, TFPI, tPA, or heparin (heparan proteoglycans) in large vessel endothelium to decrease clot propensity at atheromas or in autoimmune diseases. If systemic elevations of tPA was desired, sequence A could be used on the gene. Endogenous gene expression could be suppressed by using sequence A, ABC or possibly AC, coupled to antisense to block expression of adhesion molecules to decrease leukocyte infiltration in atherosclerosis. The thrombin response element is significantly inducible in vivo, and should therefore be particularly useful in the treatment of patients with a history of constitutively elevated levels of thrombin, for example, particularly for expression of therapeutic genes in coronary arteries in patients with unstable angina.

The present invention will be further understood by reference to the following non-limiting examples:

#### **Example 1: Isolation of Endothelium and Large Vessel Endothelium specific transcription initiator elements.**

Nucleotide sequences were determined for 8.8 kb of the genomic structure and 3.4 kb of the 5'-flanking region of the mouse EPCR (mEPCR) gene. RNase protection assay revealed six major transcription start sites clustered at -110 to -119 upstream of the translation initiation site. A series of 5'-promoter deletion fragments: mP3340, mP1120, mP700, mP350 and an SV40 control were fused to a luciferase reporter gene and transiently transfected into several cell types, bovine aorta

endothelial cells (large vessel endothelial cells), rat heart endothelial cells which is mostly capillary endothelial cells (small vessel endothelial cells), and 293 kidney cells (non-endothelial cells).

5 The results are shown in Figure 2. The expression was relatively endothelial cell specific.

Deletion of the sequence between -280 to -160 dramatically reduced luciferase expression in bovine aorta cells, as shown by Figure 3. This region of the mEPCR gene (-220 to -180) contains one AP-4 site and two overlapping SP-1 sites, as depicted in Figure 4. Mutations in the  
10 core sequence of the AP-4 site and two overlapping SP-1 sites impaired both nuclear protein binding and luciferase expression. These results indicate important roles for AP-4 and SP-1 in the constitutive expression of mEPCR.

#### **Example 2: Thrombin response element.**

15 A thrombin response element (CCCACCCC) (SEQ. ID No. 3) within the upstream region (-337 to -343) was found to mediate the induction of mEPCR by thrombin. In addition, a 380 bp fragment which spans the sequences from -1080 to -700 was identified as the endothelial cell-type specific promoter in cultured cells. This fragment could drive  
20 expression of luciferase or green fluorescent protein in large vessel endothelium but not in microvascular or capillary cells, as also shown by Figure 2.

#### **Example 3: *In vivo* Activity of the EPCR Promoter.**

Transgenic mice were developed using either the -350 to -1 or  
25 -1080 to -1 regions of the mouse EPCR promoter to drive the structural gene for green fluorescent protein (GFP) to determine the *in vivo* activity of the previously described promoter regions.

The promoter regions (-1080 and -350) of mouse EPCR gene were cloned into the pEGFP1 vector (Clontech), which already contains the  
30 structural gene for GFP. The fragments which contained the promoter region of mEPCR and GFP reporter gene were released by enzymes Eco47 III and Afl II from the constructs pEGFP350 and pEGFP1080.

After purification, the DNA fragments were microinjected into the pronuclei of fertilized mouse eggs by standard methods. Mice were screened for the presence of the transgene by GFP specific PCR and Southern blotting by standard methods. Several transgenic lines were established from both promoter constructs.

GFP mRNA was constitutively expressed in these lines. The level of GFP mRNA expression was variable from significantly less than to higher than the endogenous EPCR expression. These data indicate that the ability to express a foreign structural gene under the control of these promoters will not be chromosome integration position dependent, although constitutive level of expression may be influenced by chromosomal positioning.

**Example 4: LPS Inducibility of the EGFP1080 and EGFP350 constructs in transgenic animals**

Animals bearing the EGFP1080 construct and animals bearing the EGFP350 construct were treated with 400 micrograms LPS for 3 hours. Quantitative RT-PCR was performed to determine the level of GFP mRNA present before and after induction. GFP and mEPCR MIMICs (500 bp in length) were prepared by use of the MIMIC construction kit (Clontech). 2 micrograms of total RNA from the mice was used for synthesis of cDNA. Equal sized aliquots were then amplified in the presence of 2 microliters of a 10-fold dilution series of the appropriate MIMIC = (GFP or mEPCR). Equal aliquots were then run on a 2% ethylene bromide agarose gel. The target size is 300 bp and the MIMIC is 500 bp. The ability of the bonafide message to compete for its "MIMIC" at a particular dilution of the MIMIC indicates the abundance of the message in the original sample. Before LPS induction, the GFP mimic could not be effectively competed by the animal's mRNA until the mimic was diluted 1:100,000 for the P1080 animal and 1:106 for the P350 animal. After 3 hr treatment with 400 micrograms LPS, the EGFP1080 animal expressed at least ten times more message (mimic is

effectively competed at a 1:10,000 dilution). The EGFP350 animal could at least partially compete at the same level.

The finding that expression can be induced by treatment of the animals with endotoxin indicates that the response elements are functional  
5 *in vivo*, and with heterologous proteins.

Modifications and variations of the methods and materials described herein will be obvious to those skilled in the art from the foregoing detailed description, and are intended to come within the scope  
of the appended claims. In particular, further definition of the minimal  
10 regulatory elements using standard approaches similar to those described herein would be considered obvious equivalents.

## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: Oklahoma Medical Research Foundation
  - (ii) TITLE OF INVENTION: ENDOTHELIUM SPECIFIC EXPRESSION  
REGULATED BY EPCR CONTROL ELEMENTS
  - (iii) NUMBER OF SEQUENCES: 3
  - (iv) CORRESPONDENCE ADDRESS:
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    - (C) CITY: Atlanta
    - (D) STATE: GA
    - (E) COUNTRY: USA
    - (F) ZIP: 30309-4530
  - (v) COMPUTER READABLE FORM:
    - (A) MEDIUM TYPE: Floppy disk
    - (B) COMPUTER: IBM PC compatible
    - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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    - (A) APPLICATION NUMBER:
    - (B) FILING DATE: 07-NOV-1997
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  - (vii) PRIOR APPLICATION DATA:
    - (A) APPLICATION NUMBER: US 60/030,718
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  - (viii) ATTORNEY/AGENT INFORMATION:
    - (A) NAME: Pabst, Patrea L.
    - (B) REGISTRATION NUMBER: 31,284
    - (C) REFERENCE/DOCKET NUMBER: OMRP 164 PCT
  - (ix) TELECOMMUNICATION INFORMATION:
    - (A) TELEPHONE: 404-873-8794
    - (B) TELEFAX: 404-873-8795
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 3360 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: DNA (genomic)
  - (iii) HYPOTHETICAL: NO
  - (iv) ANTI-SENSE: NO
  - (ix) FEATURE:
    - (A) NAME/KEY: misc\_feature
    - (B) SOURCE: murine
    - (D) OTHER INFORMATION: /note= "Nucleotides 2270 through 2840 are  
a large vessel endothelial specific element".
  - (ix) FEATURE:
    - (A) NAME/KEY: misc\_feature
    - (B) SOURCE: murine
    - (D) OTHER INFORMATION: /note= "Nucleotides 2990 through 3061 are  
a serum response element".
  - (ix) FEATURE:
    - (A) NAME/KEY: misc\_feature
    - (B) SOURCE: murine
    - (D) OTHER INFORMATION: /note= "Nucleotides 3007 through 3014 are  
a thrombin response element".
  - (ix) FEATURE:
    - (A) NAME/KEY: misc\_feature
    - (B) SOURCE: murine
    - (D) OTHER INFORMATION: /note= "Nucleotides 3130 through 3350 are  
an endothelial specific element".
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTTACT CTGCCACATT TCTTCTGCCC GGCCAGGAT GTAGGCCTCT TTATTAACCA	60
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 AAATTTCTTA TTCTGCTCTA CTTCAATTTT TCAAAAGACA TACTAATTG TGATGTCATT 2700  
 GCCACTAAAT GACTATGACC TGTCCGATGC TGAGATTTAT CTAGAGCGTT CCTAAATCTC 2760  
 TGCCACAATG AACTCTTTTT TACTCACTCG ACTCTGTGAC TATTTCTGAG AGCCCTCTC 2820  
 CTCCAGTTGT GTAATTCCTG TGTACTTAAA CTTCTGATAA ACTATAGGCA GTTATCCTGG 2880  
 AAAGTTAGAT TCCAATCCTG GATCTGCCGT CATCGGGACG TACAACTTT GGGCAAATCC 2940  
 CTACATCTCT TTTGACCTCA GTTCCCCGT CATCTCTACA GAGTCGGCAA CATCGAAAGC 3000  
 AGACGCCCCA CCCCCCTGAC TCAGCGGCGA CCTACCGGAC TTCTCGCCAA GCCCTTCTCC 3060  
 CCCTTTTCCG CTCCTCCTCA AGCCTCGGAA GCAAGCAGCG GGAGGAGAAA CAGGCAGGTC 3120  
 CAGGCAGGAG GGCCACAGC TGGGAGGGGCG CGAGGCGAGC CGGCCCCCTA GTAGGAAATG 3180  
 AGACAGATCC AAGTAACACT TTAAAAGCCT GACTCCCTCT TCCTGCACGC GTTCTCTTTC 3240  
 CATCTCCGC TCTGCTCCGG CCCCTCCCGG ACAGCCTCCC TTCTCTTCC TAATCAGCAG 3300  
 CCTGAGGAAC CCGAGCCTGC CCCGACCCAG GTGGGACCCA GAACTCCAGG ATGTTGACGA 3360

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3097 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) SOURCE: Human
- (D) OTHER INFORMATION: /note= "Nucleotides 2722 through 2729 are

a thrombin responsive element".

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTGGAAGAAA ACTTAAGTGT TCAGCAACAA GAGAATGGAT ACATAAATAA TGATCTATTC 60  
 CCAAAATTGA TTTTTTTTTT TGAGACAGGG TCTTGCTCTG TTGCCAGGC TAGAGTGCAG 120  
 TGGCATGATC ATGGCTCACT GCAGTCTCAA CCTCTGGGC TCAAGCAATC CTCCTACCTC 180

AGCCTCCTGA GTAGCTGAGA CCACAGGCAC AACCCATCAC ACCCAGCTAA TTTTATTTTT	240
TTTGTAGAGA TGGGGGTCTC ACTATGTTGC CCAGGCTGGT CTGAACTCC TGGGCTCAAA	300
TGATCCACCC ACCTCGGCCT CCAAAGTGCT GGGATAATAC CTCCCAGCC GGATATTTTA	360
AAGCAGTGAA AATGAATGGT CTACACATAG CCACATGAAT GAATCTTATT AATACATTAA	420
GTGAAAAAAA GCAAAGGTCA CAGAGGAATA CATACATTTT AATACCATTT ATATAAAGCT	480
CAAAATATGT GAAATACCAC TATCTATTGT TTAGGGATAT ATACATAAGT AGTGTAAGTA	540
TACAGAAATA TAAGGAAATG AAAAATATCA AATCTTCATT TTCATCTGAA GTGGTTACTT	600
CAGGGGCTGT GGCAGGGAGA GAGAGATGCA GCTGAGGAAG AGTCCATAGG GGGCTTCAAC	660
TATATTAGCA ATATTGTATT TCTTATGCTT GGTGGTGGGG ATAGGTATGT TTGAAATGTA	720
ATCCTTTAAG CATGAAATAA CTCTTCAAAA ATGAAATATT TCAGGCTGTG CACAGTGGCT	780
CAGGCATTGT AATCCCAGCA TGTTGGGAGG CTGAACGTGG GCGGATCACC GTGAGGTCAG	840
GAGTTTGAGA CCAACCTGGC CAACATGGTG AAATCCCATC TCTACTAAAA ATACAAAAAT	900
TAGCCAGGTG TGGTGGCAGG TGACTGTAAT CCCAGCTACT TGGGAGGCTG AGGCAGGAGA	960
ATCGCTTGAA TCTGGGAGGT GGAGGTTGCA GTGAGCCGAG ATCAGCCGAC TGCATTACAG	1020
CAAGACTCCA TCTCAAAAAA AAGAAAAAAA AAAAGAAAAA AGAAATGTTT CATAATTTTT	1080
AATAAAAGGC AAGACAATAT AAATTGGTAG TTATTTAAGT CATTCTACTT TTCCTGAGGC	1140
CCAGTGCAGG AAAACAAAGT TCCTATCCTT GTTCCAATA GACCATTTTG ATAAGCTGCA	1200
AAAAGAAAAG ACTTTGATGC TATTTCTTAG CCAGTTTGCA ACAGCTGAGA GGTGAGCATG	1260
GAAGCTCTTG CATATATTCA GTTCAGAGAA TGGGTGCTTA GTTTATGTCC AGAGTTTGTC	1320
CCAGATTTCA CTATGACGTC AGCTCTCCGG GGAGAAGTAT ATAAAATAAA AAGTTAAAT	1380
CCCTCTCAGT CCTTTACCCA ATCCTATTCC CCAGAGGTAA TCTCTATTGA CAGTACCCCT	1440
CCAGATATTT TCCCTATGTA TATACAAATA CACAGATACA CACTGAAAGT TAATTTTGGC	1500
CAGGTGCAGT GGCTCCTGCC TATACCAGAG GATTGCTTGA GTGCAGGAGT TCAAGACCAG	1560
CCTGGGCAAC ATAGCGAGAC CACATCTCTA GTAAAAATAA AAAAAAATAG CTAGGCGTGG	1620
TGGCACAGTG GCACGTACCT TTAGTCTCAG CTACTCGGGT GGTGAGGTG GAGAATCACT	1680
TGAGCCCGGG GAGGTCAAGC CTACAATTAG CTGTGATTGC TTTACTGCAC TATAGCCTGG	1740
GCAACAGAGC TAGACCCTGT CTCAAAAAAA TAATAATAAA TTTTATATAT ATATATGAGG	1800
ATGAAATTAC ATATGTATTA TTTGAACAGA AGTGAAATCT TTTCTTTTTT TTTTCAGAC	1860
AGAATCTTGC CGCATGACCC AGGCTAGAAT GCAGTGGTGT GATCTCGGCC CTCTGCAACC	1920
TCCACCTCCC AGGTTCAAGC GATTCTCATG CCTCGGTCTC CCAAGTAGCT GGGATTACAG	1980
GCATGCACCA CCATGCCAG CTAAATTTTG TATTTTTCGT AGAGACGTTT GCCATATTGG	2040
CCAGGCTGGT CTCAAATCC TGGCCTCAAG TGATCTGCCC ACCTCGGCCT CCCAAAGTGC	2100
CAGCAGCATG CTCGGAGGAG TGACTTTAAA GCTTTTCTAC TTGCTTCCTA GAGTAAGGGA	2160
CGCATTTTAC ACTGCTATCC AAAACTCATC ATAGAAACAT ACACACACAA AACCAAAGCA	2220



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CACATATACA ACTGAGCAAA TATTTTCATGA CATAACACTT TCTCTTACTA AGGGTGACGC	2280
GCTGAAATTT TGTATTCTGT CCTATTTTCAT TTTTAAAAA TGGAACCAT GACCTGCTAA	2340
ATTGATTTCA TTGTCCACTA ATAAATTATG ACCTCAGTTT CAAAAAGATT GCTTTAGGTA	2400
ACCAATCATC TTCTGAGATT TATACAGATT GCTCATAATT CTCTCCTATT TTTTAAAAAC	2460
ATGCTGCAGT GAACTGCTTT AACTCATT TATGACTACT TCTGAGACCA AGATCCCGGA	2520
TTATGTAATT GTTATTTACT TAAAATTCTG GTAAATGTA GCCATTATAC TGGAAAACTA	2580
AATTTTAATC TTGGATCTGT CACCACCATG ATATATAAAC TTTGGGCAAG TCCCTGCACC	2640
TCTCTGGACC TCAATCTCCC CATCAGCAAC CTGCTGATCC TACTCCCAGG AGTGTGCTCT	2700
AAGTTGAAAG TAGATGCCCC ACCCCCTGAG TCAGCGCCGG CAGGACTTCT CACCAAGCCC	2760
TTCTCCCCCT TTTCCGCTCC CTGTTCTTGG TTCCTAGGAA GCAGCCCAAG GAGAAGGGAA	2820
AAGGCAGGTC TGGGCAGGAG GGAGCAATGA AGGGCGGGC AGAGGGAGGG CAGGAGGGAG	2880
GCCGGCCCCC TAGTAGGAAA TGAGACACAG TAGAAATAAC ACTTTATAAG CCTCTTCCTC	2940
CTCCCATCTC CTGGCCTCCT TCCATCCTCC TCTGCCCAGA CTCCGCCCCT CCCAGACGGT	3000
CCTCACTTCT CTTTTCCCTA GACTGCAGCC AGCGGAGCCC GCAGCCGGCC CGAGCCAGGA	3060
ACCCAGGTCC GGAGCCTCAA CTTCAGGATG TTGACAA	3097

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCACCCC

We claim:

1. An isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells.
2. The element of claim 1 depicted in SEQ. ID No. 1, between nucleotides 3130 and 3350.
3. The element of claim 1 depicted in SEQ. ID No. 2.
4. An isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells.
5. The element of claim 1 depicted in SEQ. ID No. 1, between nucleotides 2270 and 2840.
6. The element of claim 1 depicted in SEQ. ID No. 2.
7. An isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum.
8. The element of claim 1 depicted in SEQ. ID No. 1, between nucleotides 2990 and 3061.
9. The element of claim 1 depicted in SEQ. ID No. 2.
10. A construct for expression of a heterologous gene comprising a regulatory element selected from the group consisting of an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells; an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells, and an isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum.
11. The construct of claim 10 further comprising a heterologous gene.
12. The construct of claim 10 further comprising a thrombin response element depicted in SEQ ID No. 3.
13. The construct of claim 10 comprising an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells and an isolated regulatory element

present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells.

14. The construct of claim 13 further comprising the thrombin response element depicted in SEQ ID No. 3.

15. A method for controllably expressing a gene or biologically active nucleotide molecule comprising expressing the gene or biologically active nucleotide molecule under the control of a regulatory element selected from the group consisting of an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells, an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells, and an isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum.

16. The method of claim 15 wherein the gene is also expressed under the control of a thrombin response element depicted in SEQ ID No. 3.

17. The method of claim 15 wherein the gene is expressed under the control of an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells and an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells.

18. The method of claim 15 wherein a gene encoding a protein is expressed.

19. The method of claim 15 wherein a biologically active nucleic acid molecule selected from the group consisting of antisense, triplex forming molecules, ribozymes, and guide sequences for RNAase P, is expressed.

20. The method of claim 15 wherein the gene or biologically active nucleotide molecule is expressed in a patient in need of treatment thereof.

21. The method of claim 15 wherein the gene or biologically active nucleotide molecule is expressed in cell culture.

FIG. 1A

3273 AG.CCTCCCTTCTCTT..CCTAATCAGCAGCCTGAGGAACCCGAGCCTG 3319  
| | | | | | | | | | | | | | | | | | | | | |  
3001 GGTCCCTCACTTCTCTTTCCCTAGACTGCAGCCAGCGGAGCCCGCAGCCG 3050  
3320 CCCCAGACCCAGG.....TGGACCCAGAACTCCAGGATGTTGACGA 3360  
| | | | | | | | | | | | | | | | | | | | | |  
3051 GCCCGAGCCAGGAACCCAGGTCGGAGCCTCAACTCAGGATGTTGACAA 3100  
3361 AGTTTCTGCCGCTACTGCTGCTGCTGCCCTGGCTGCCCCCTTTGTA.. 3408  
| | | | | | | | | | | | | | | | | | | | | |  
3101 CATTGCTGCCGATA.....CTGCTGCTGCTGGCTGGCCCTTTGTAGC 3144  
3409 .....ACTCCGATGGTGAGTTTGGGTCAAGGCTCCTGCCCTGGGGGT.G 3450  
| | | | | | | | | | | | | | | | | | | | | |  
3145 CAAGACGCCCTCAGATGGTGAGTCGGGGGCACATCTCCTGCCCTCAGGATGG 3194  
3451 TTCTAGGA..CTTGGTGATTTGGGAACCTTG 3481  
| | | | | | | | | | | | | | | | | | | | | |  
3195 TTCTGGAGAATCTCAGTCTATCTGGGCACATGG 3227

FIG. 1B

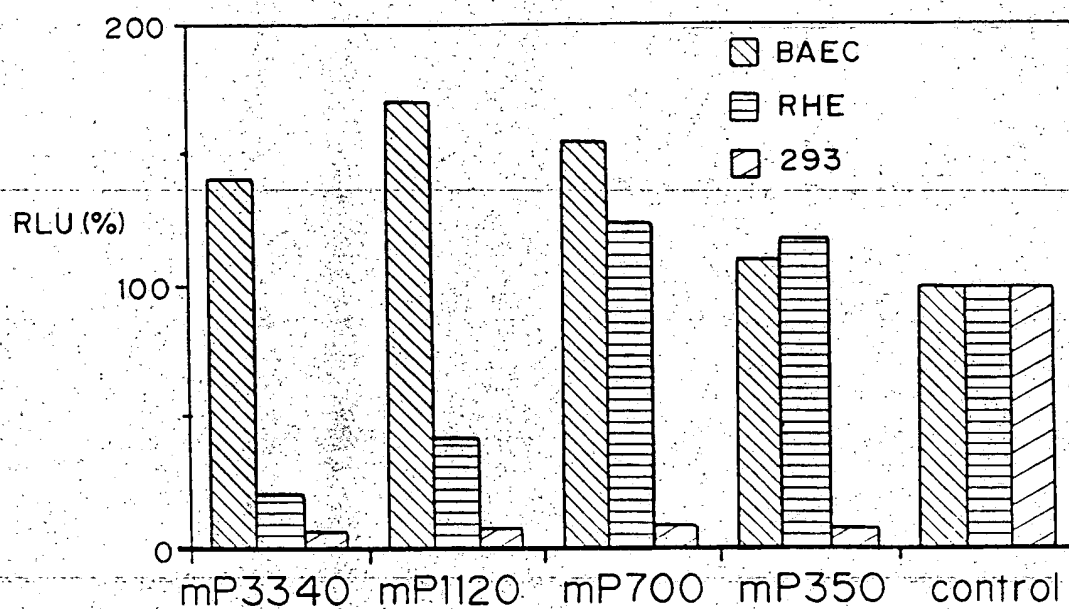
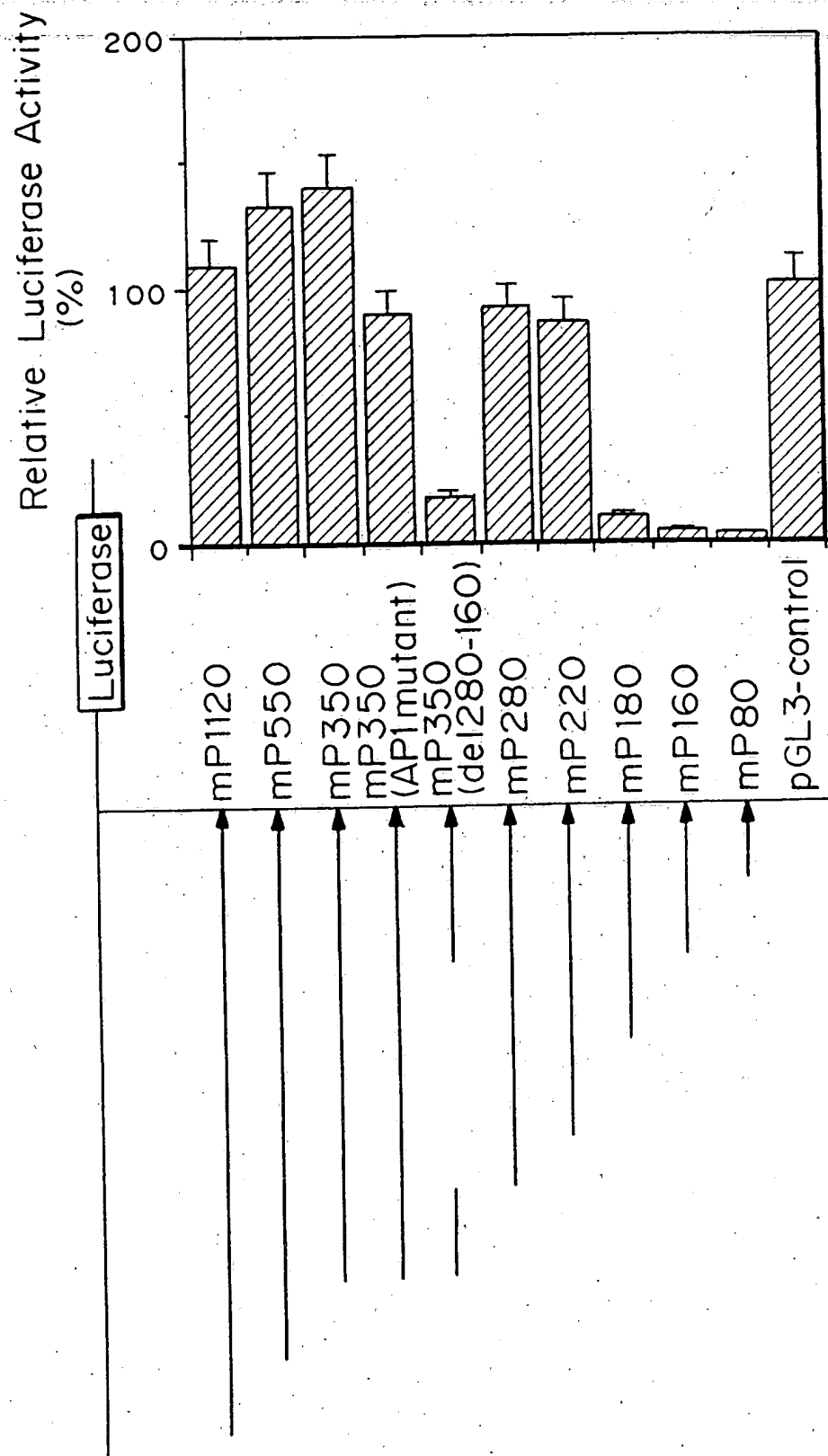


FIG. 2

FIG. 3



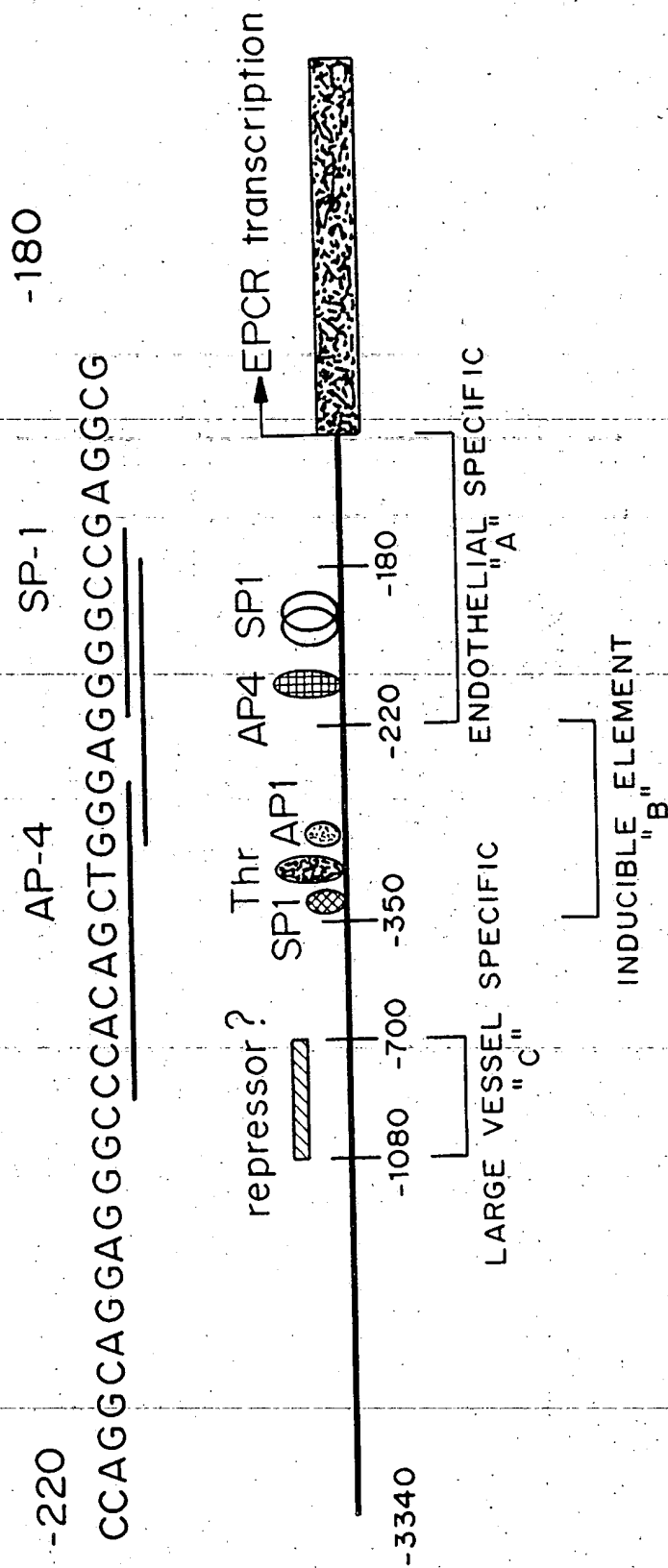


FIG. 4



# INTERNATIONAL SEARCH REPORT

Internat: Application No  
PCT/US 97/20364

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. FUKUDOME AND C.T. ESMON: "Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR)" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 10, 1995, BETHESDA, MD, US, pages 5571-5577, XP002058876	1,4
Y	see the whole document and specially figure 1	12,16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

23 March 1998

Date of mailing of the international search report

07.04.98

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# INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/US 97/20364

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category <sup>2</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	W. DING ET AL., : "Upregulation of the message for rodent endothelial cell protein C receptor (EPCR) by endotoxin and thrombin" CIRCULATION, vol. 94, no. suppl. 8, 15 October 1996, NEY YORK, NY, US, page I-694 XP002058206 see abstract	12,16
X	EMBL sequences database, HEIDELBERG, DE M. Marra - 9 August 1996 Mus musculus cDNA clone similar to centrocylin The WasU-HHMI-mouse EST project XP002058879 Accession number: AA016910 see abstract	1,2
X	D. SEMON ET AL., : "Nucleotide sequence of the murine TNF locus, including the TNF-alpha (tumor necrosis factor) and TNF-beta (lymphotoxin) genes" NUCLEIC ACIDS RESEARCH, vol. 15, no. 21, 1987, OXFORD, GB, pages 9083-9084, XP002059704 see the whole document	5
X	S.M. GARDNER ET AL., : "Mouse lymphotoxin and tumor necrosis factor: Structural analysis of the cloned genes, physical linkage, and chromosomal position" JOURNAL OF IMMUNOLOGY, vol. 139, no. 2, 1987, BETHESDA, MD, US, pages 476-483, XP002059705 see figure 3	5
X	A. PELERAUX ET AL., : "Genomic organisation of a mouse MHC class II region including the H2-M and Lamp2 loci" IMMUNOGENETICS, vol. 43, 1996, NEW YORK, NY, US, pages 204-214, XP002059706 see figures 3A,B	5
X	WO 96 05303 A (OKLAHOMA MED RES FOUND) 22 February 1996 cited in the application see abstract see page 27, line 13 - page 31, line 21 see page 14, line 6-30	15,16,20
1	---	

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# INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/US 97/20364

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 94 04694 A (MOUNT SINAI HOSPITAL CORP) 3 March 1994  see page 1, line 32 - page 2, line 3 see page 14, paragraph 3 - page 15, paragraph 2 ---	1,4,10, 11,13, 15,17,18
A	WO 93 09236 A (BAYLOR COLLEGE MEDICINE) 13 May 1993 see abstract see page 11, line 27 - page 12, line 5 ---	7,10,15
A	N. BANGALORE ET AL., : "High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells" THROMBOSIS AND HAEMOSTASIS, vol. 72, no. 3, 1994, STUTTGART, DE, pages 465-474, XP002059708 see the whole document ---	10,11,15
P,X	EMBL sequences database, HEIDELBERG, DE M. Marra - 25 July 1997 Mus musculus cDNA similar to M. musculus endothelial cell activated protein C recep tor. The WashU-HHMI mouse EST project XP002058878 accession number: AA389039 see abstract ---	1,2
P,X	EMBL sequences database, HEIDELBERG, DE M. Marra - 14 December 1996 Mus musculus cDNA clone The WashU-HHMI mouse EST project XP002058880 Accession number: AA146223 see abstract ---	4,5
T	J.M. GU AND C.T. ESMON: "Functional characterization of the 5'-flanking region of the mouse endothelial protein C receptor (EPCR) gene" MOLECULAR BIOLOGY OF THE CELL, no. suppl. 8, 15 December 1997, BETHESDA, MD, US, page 228a XP002058877 see abstract -----	1,4,11, 13,17, 18,21

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat. Application No.

PCT/US 97/20364

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9404694 A	03-03-94	US 5466596 A CA 2143336 A EP 0658209 A JP 8500247 T	14-11-95 03-03-94 21-06-95 16-01-96
WO 9309236 A	13-05-93	US 5298422 A AU 660751 B AU 3124693 A CA 2122617 A EP 0635060 A JP 7500967 T PT 101042 A	29-03-94 06-07-95 07-06-93 13-05-93 25-01-95 02-02-95 28-02-94